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PATHOPHYSIOLOGY OF PEPTIDE TOXINS OF MICROCYSTIS AERUGINOSA  
AND AMANITA PHALLOIDES

FINAL REPORT

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Studies focussed on the pathophysiology of the cyclic peptide toxin from M. aeruginosa and prophylaxis against it. The critical role of the liver as a target organ for the toxin and site of prophylaxis was established. The particular liver cell type critical to the toxin was not established. The most effective protective agents were trypan red and cyclosporine. The mechanism of action of protection was not established, but the low effective doses for each protective agent suggested great specificity in the mechanism of protection. *Keywords: Antitoxins Phytotoxins, (AW)*

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# FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).



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## STATEMENT OF PROBLEM UNDER STUDY

Blue-green algal toxins include small cyclic peptides which are known to be lethal to mammals and birds either by ingestion of algal blooms or by parenteral injection (1-4). While their toxicity in humans has not been documented, there is some evidence that gastroenteritis and liver disease can result from drinking water which has been heavily overgrown by the algae (5,6). Nevertheless, the virulence of a purified cyclic peptide toxin such as toxin-LR\*, a heptapeptide of Microcystis aeruginosa, is equivalent to that of the highly poisonous toxin,  $\alpha$ -amanitin, a cyclic octapeptide of the mushroom, Amanita phalloides, which annually is the cause of several hundred deaths worldwide (7).

Earlier studies from this laboratory have characterized several features of toxin-LR pathophysiology, as well as some methods of prophylaxis against acute toxin-LR lethality in mice (8,9). Furthermore, the methods were found to be equally effective against the A. phalloides toxin, phalloidin. With these observations at hand and with the M. aeruginosa cyclic peptide, toxin-LR, available for use as a probe (10), studies

\* The recently suggested designation for this and related M. aeruginosa toxins is "cyanoginosin", in which case toxin-LR would be termed cyanoginosin-(LR) (11). In this report, however, the term toxin-LR will be retained for the cyclic heptapeptide in which the two variable amino acids are leucine (L) and arginine (R) whose molecular locations are unspecified.

were undertaken to extend the list of chemical agents capable of blocking the toxin and to define the mechanism for protection.

To better understand how protection against toxin-LR and related low molecular weight toxins could be accomplished, concurrent studies were planned to define more clearly the pathophysiology of toxin-LR and the effects of structurally related cyclic peptide toxins of M. aeruginosa.

#### BACKGROUND

The cosmopolitan cyanobacterium, Microcystis aeruginosa, frequently a major component of freshwater cyanobacterial blooms, contains potent lethal peptides which are composed of three invariant D-amino acids (Ala, erythro- $\beta$ -methyl-D-iso-Asp, and Glu), two variant L-amino acids, N-methyl dehydroalanine, and a beta-amino acid (12). Multiple toxins have been purified from clonal isolates. The toxic peptide used in the present investigation is denoted toxin-LR using the standard one-letter abbreviations for its two variant amino acids, leucine and arginine.

Earlier reports stressed the importance of the liver as the target organ for M. aeruginosa peptides, and hepatotoxicity was believed to be the primary cause of death in animals injected with either the purified or crude toxins (13). Slatkin et al.

(8), in studies of the effects of toxin-LR in Swiss mice, questioned this interpretation, noting that death from the injected toxin occurred so rapidly that hepatotoxicity, even if present, was unlikely to be a significant contributing factor. Occlusive pulmonary vascular lesions were also confirmed as a consequence of toxin-LR injection (Fig. 1). Slatkin et al. initially interpreted the lesions to be atypical thrombi and that the small, globular structures present in the lesions included platelets or leukocytes (8). It was hypothesized that extensive pulmonary artery occlusion by the thrombi produced acute right-sided heart failure and death. This sequence of events seemed logical since it was discovered that an abrupt decrease in platelet count and an increase in liver weight developed concurrently with the atypical thrombi. Subsequent study, however, revealed that even though platelets accumulate in massive numbers in the liver following the injection of mice with toxin-LR (Fig. 2), irradiated mice made severely thrombocytopenic and leukopenic were just as susceptible to toxin-LR lethality as were normal mice (9). Furthermore, anticoagulants and antiplatelet drugs provided no protection against the toxin. Apparently thrombus formation and platelets were not important etiologic factors in toxin-LR pathophysiology. However, it was conceivable that certain vasoactive substances might play a role. Those substances include derivatives of arachidonic acid, such as the thromboxanes, prostaglandins, and leukotrienes.

Hydrocortisone is known to block the formation of

arachidonic acid from membrane lipids and thus interferes with the production of both the prostaglandins and leukotrienes (14). Hydrocortisone in pharmacologic doses prevented toxin-LR lethality as well as the hepatic enlargement and thrombocytopenia (9). However, other substances which interfere with arachidonic acid metabolism, such as ibuprofen and aspirin, did not ameliorate toxicity (8). Another effect of glucocorticoids and one which requires large doses is reticuloendothelial blockade (15), which may be defined as impaired plasma clearance of colloidal particles. As a consequence, India ink, which has been known for 60 years to alter macrophage function (16), was used to induce reticuloendothelial blockade.

When India ink (No. 4415, Faber-Castell Corp., Newark, NJ) was injected intravenously into mice, protection against toxin-LR developed immediately and persisted for 1-2 days. Thus further credence was given to the hypothesis that the reticuloendothelial system was critical to some aspect of toxin-LR activation or mechanism of action, and that protection could be induced by modulating macrophage function. Since the liver was the target organ for toxin-LR-induced injury, the macrophages which would be particularly relevant were the hepatic Kupffer cells.

A previous report indicated that neonatal mice were resistant to M. aeruginosa toxins (17). This observation was confirmed (9), and development of sensitivity to toxin-LR was more precisely determined to occur at about the 18th day after birth (unpublished data). Some hepatic enzyme systems are not



fully developed at birth, and it was hypothesized that the liver was involved in activating toxin-LR. To test this idea, adult mice were administered hepatotoxic doses of carbon tetrachloride. Beginning 24 hours after injection of carbon tetrachloride the mice were protected against toxin-LR and the protection persisted for one week (9). The return of sensitivity to the toxin presumably correlated with hepatic regeneration. Efforts to determine the metabolic pathways that might be involved in toxin activation were unsuccessful. Neither SKF-525A, an inhibitor of many cytochrome P-450 enzymes, nor 2,4-dinitrophenol, an uncoupler of oxidative metabolism, altered the lethality of toxin-LR in mice.

#### RATIONALE USED IN THE CURRENT STUDY

Preliminary studies suggested that the liver was critical to the development of the pathophysiologic effects of toxin-LR in addition to being the target organ. A research protocol was devised which would concentrate on the role of hepatocytes, Kupffer cells, and endothelial cells in determining both the acute and chronic toxicities of toxin-LR and the mechanism(s) of prophylaxis. A search for other forms of protection would center around those agents that interfered with reticuloendothelial function. Complementary studies on the pathophysiology of toxin-

LR were also planned. As in any basic research effort, it was understood that a redirection of the proposed plan might be desirable if new discoveries suggested more profitable avenues of relevant research.

## EXPERIMENTAL METHODS

1. Experimental animals: Adult Swiss mice of the Brookhaven National Laboratory Hale-Stoner strain were used for most studies. Female or male C-57 and Swiss-Webster mice from the Laboratory breeding stock or from Taconic Farms (Germantown, NY) were used to evaluate strain differences. When it was determined that responses of all three strains to toxin-LR were similar, C-57, Swiss-Webster, and male Hale-Stoner mice were used in some experiments requiring large numbers of animals. Standard caging was provided and Purina rodent chow was available ad libitum except for studies of toxicity following administration of toxin-LR by gavage. All animals were maintained at  $22 \pm 1^\circ \text{C}$  and  $55 \pm 5\%$  humidity with 12 hr (7 a.m. to 7 p.m.) of fluorescent light per day. In the long-term studies each mouse was identified by a numbered ear tag, examined weekly for pathophysiological changes, and weighed monthly. Limited studies were performed using Sprague-Dawley rats (Taconic Farms) since large amounts of toxin are required per animal to produce death.

## 2. Toxin preparation:

a. Strain origin. Thirty strains of M. aeruginosa obtained from Europe, Africa, Australia, Japan, the Middle East, and North America are maintained in our culture collection. They are held on agar slants and in 5% dimethyl sulfoxide at  $-80^{\circ}$  and  $-196^{\circ}$ . Low temperature storage minimizes potential genetic variability and microbial contamination. Axenic cultures are derived by streaking on 1% nutrient agar and selection of individual clones for subculture. Slow growth on agar and difficulties in eliminating microbial contaminants impede obtaining axenic strains. Contamination is monitored on 1% nutrient agar plates. Liquid and slant cultures are easily contaminated despite maintenance of good sterile practices. All transfers are made in a transfer hood.

b. Cultures. Cell cultures are initiated in 250 ml flasks. In about two weeks, the culture is transferred to a 1 l flask and aeration with 0.1%  $\text{CO}_2$  in air is initiated. After about 2 weeks, the culture is transferred to an 8 l bottle with 0.1%  $\text{CO}_2$  in air aeration. The light intensity during these operations is about  $50 \mu\text{E m}^{-2} \text{sec}^{-1}$  (cool white fluorescent lamps). After about 1 week, the culture is transferred to 4 - 20 l bottles, aerated with 1.0%  $\text{CO}_2$  in air, and illuminated with about  $100 \mu\text{E m}^{-2} \text{sec}^{-1}$ . The 20 l cultures are transferred after about 1 week to an

illumination of about  $300 \text{ uE m}^{-2} \text{ sec}^{-1}$ . The culture densities are monitored spectrophotometrically and harvested at a cell density of about  $A = 3 \text{ cm}^{-1}$  at 725 nm after about 1 week at the highest light intensity. Yields of cells from a 20 l bottle vary from 0.5 to 3.0 g l<sup>-1</sup> are strain dependent. Some strains produce volatile products which can be offensive to sensitive individuals, and exiting air from all large cultures is exhausted outside the building. Culture temperature is usually 28°. One kg lots of cells can be harvested monthly. Maintenance of a culture collection and growth of M. aeruginosa under sterile conditions is demanding of time and labor intensive. As an example, preparation of 200 l of sterile media is required weekly to maintain kg harvests every month.

c. Toxin purification. Each strain of M. aeruginosa used in these studies contains a unique complement of toxins. Isolation of the toxins from each strain has unique problems associated with the biochemical composition of the cells.

Toxins are isolated from kg lots of cells by repeated extraction with 25% acetonitrile - 10 mM ammonium acetate, pH 6.0 or 25% methanol - 10 mM ammonium acetate, pH 6.0. The extracts are clarified by centrifugation and concentrated by rotary evaporation with Dow antifoam and n-butanol to avoid foaming and to promote smooth evaporation. Concentrates of the first 2 or 3 extracts are frequently highly viscous due to their content of either storage polysaccharides or lipopolysaccharide wall

components. The viscous concentrates are unsatisfactory for chromatographic purification. Adjustment of these extracts to about pH 9 with sodium hydroxide and addition of 10 mM calcium chloride and 1 mM cetyl trimethyl ammonium bromide precipitates a portion of the polysaccharides. However, complete removal has not yet been achieved by precipitation methods. Small amounts of polysaccharide are currently removed by diluting concentrated extracts, to reduce viscosity, and gel chromatography on 5X20 cm columns of Fractogel HW-40-F with 5% 2-ProH - 10 mM ammonium acetate, pH 6 as eluting solvent. Toxic fractions are partially separated on the Fractogel column. The partially purified toxins are then purified by preparative HPLC on 1.0 X 25 cm or 2.5 X 25 cm columns of octadecyl silica (30  $\mu$ m particle size, Serva Fine Biochemicals). The solvent system used is a stepwise gradient increasing from 40 to 50% methanol - 0.05 M ammonium acetate, pH 6 at 5 or 10 ml min<sup>-1</sup>. Further separations were obtained with 20 to 30% acetonitrile - 0.5M ammonium acetate, pH 6 if required. All fractionations were monitored by HPLC with a 0.46 X 5 cm column of octadecyl silica gel (3  $\mu$ m particle size, Shandon) using 27% acetonitrile - 0.5 M ammonium acetate, pH 6 at 240 nm. The analytical HPLC procedure can detect 2% of a lethal mouse dose (about 50 ng of toxin) and detection may be decreased to about 0.2% if required.

d. Safety precautions. During all stages of cell culture, care is take to avoid direct contact with the cells. No problems of

allergic reaction were observed. Volatile emanations from certain cultures have caused nausea and possible reactions in selected individuals. The outgoing airstream from large cultures is exhausted through the fume hood system.

Care is taken during toxin purification, especially with concentrated preparations, to avoid skin contact. Chromatographic solvents are prepared and stored in a fume hood, and their evaporation is minimized during use.

e. Standardization of toxins. Following separation on preparatory columns the concentration of the undiluted toxin is approximately 1.0  $\mu\text{g}/\mu\text{l}$ , based on a chromatographic standard previously established. For each lot of concentrated toxin the minimal  $\text{LD}_{100}$  dose of toxin-LR is determined. Mice are challenged intravenously with toxin-LR diluted in 0.9% saline solution and injected in dose increments of 0.001 ml/gm body weight (gbw). Acute deaths are obtained within a period of 50-120 min. The  $\text{LD}_{100}$  of toxin-LR is consistently 0.1  $\mu\text{g}/\text{gbw}$ . Subsequent studies of two related cyclic peptide toxins, toxin-LA and toxin-RR, have shown  $\text{LD}_{100}$  doses to be apparently about the same as for toxin-LR.

f. Routes of toxin-LR administration. Earlier studies had shown that there was little difference in the pathology or time of death when the toxin was administered either ip or iv. It was proposed that respiratory and gastrointestinal exposures would

also be evaluated. Respiratory exposure was carried out by intratracheal instillation. This was done because defined doses of the toxin would be delivered reproducibly to each animal, less toxin would be required per animal, the technique is simpler to perform, and a pulmonary dose-response curve can be determined more accurately. Thus, a transoral tracheal intubation technique utilizing a fiberoptic laryngoscope was used. Tracheal tubes were constructed of 0.036 in PEG tubing. A plastic barb was attached to limit tracheal penetration to 25 mm, measured from the incisors. An adjustable Finnpiquette (50-250  $\mu$ l) was modified to accept a 24 ga 3/4 in needle. With this apparatus, 30-90  $\mu$ l of toxin solution followed by a 200  $\mu$ l air bolus was delivered to the trachea.

For study of gastrointestinal administration, standard gavage procedures were used.

3. Routine tests: Platelet counts on mice were performed on blood obtained from the tail vein. Specimens were collected and diluted using the Unopette system (Becton-Dickinson Co., Rutherford, NJ). Other blood cell counts were performed on an electronic particle counter. Platelets were counted using phase microscopy, allowing at least 30 min for settling in the hemocytometer chamber. Coagulation tests included the thrombin time (Topical Thrombin, Parke-Davis, Morris Plains, NJ), prothrombin time and partial thromboplastin time (Dade Diagnostics, Aguado, PR). Biochemical tests, including tests of

hepatic and renal function, were performed on a Technicon Autoanalyzer (Technicon Corp., Tarrytown, NY). Serum protein electrophoresis was carried out on cellulose acetate strips (Helena Labs, Beaumont, TX) and quantitated densitometrically. Routine tissue samples were fixed in buffered formalin and stained with hematoxylin/eosin. Glycogen in sections was histochemically determined using Best's carmine stain.

#### 4. Hepatic studies:

a. Serial histological, histochemical, and blood chemistry studies. The temporal changes which toxin-LR produced in hepatic and pulmonary histology, blood chemistry values, and circulating platelets were determined. Female Hale-Stoner mice, 6 wk of age, were killed at 5 min intervals (up to 30 min) after an iv LD<sub>100</sub> dose of toxin-LR. Liver, lungs, spleen, kidneys, heart, and thymus were removed, weighed, fixed, sectioned, and stained. Section of liver and lung were also stained for glycogen with concurrent diastase digestion controls. Heparinized blood samples for biochemical testing were obtained by heart puncture on ether-anesthetized animals.

b. Hepatectomy. It was originally intended that the importance of the liver in activating toxin-LR would be performed on partially hepatectomized rats. However, to conserve the supply of toxin, hepatectomies were performed on mice. This procedure is difficult with mice because of their small size, the fragility of the liver, and their sensitivity to ether anesthesia. The



procedure used has permitted the removal of 60-85% of the liver, although the mortality of the procedure is about 50%. Twenty-four hr after surgery the surviving animals were challenged with an LD<sub>50</sub> dose of toxin-LR.

5. Reticuloendothelial system studies: Assays of reticuloendothelial function based on particle clearance from the blood usually take several days for completion. In the present studies, it was important to determine the kinetics of macrophage impairment. An assay was selected which permitted an in vivo assessment in only two hours by measuring the clearance of <sup>51</sup>Cr-labelled sheep red cells (18). Five ml of sheep red cells preserved in Alsever's solution (Gibco Diagnostics, Lawrence, MA) were incubated at room temperature for 30 minutes with 100  $\mu$ Ci of <sup>51</sup>Cr-Na<sub>2</sub>CrO<sub>4</sub> (New England Nuclear, Boston, MA). After three saline washes, a 0.1 ml aliquot containing 10<sup>7</sup> red cells was injected via the tail vein into test mice. Preliminary studies indicated that a larger number of cells decreased the sensitivity of the test, which depends on the ratio of spleen to liver radioactivity two hours after injection. At the conclusion of the assays the liver and spleen were removed, weighed, and counted for total organ radioactivity in a gamma scintillation counter. The percent uptake of the injected dose by the spleen was divided by that of the liver. An increase in this ratio indicated a blockade of macrophage function, primarily in the liver. Sequential assays were performed in mice given protective

agents, permitting a correlation between duration of protection and evidence of impaired reticuloendothelial function.

Reticuloendothelial activity varies with sex, females generally having greater levels (19). Therefore, the comparative susceptibility to toxin-LR was studied in both male and female Hale-Stoner mice. To determine if estrogens could alter the response, conjugated estrogens (Premarin, Ayerst Laboratories, Inc., New York, NY,) were injected into mice 1, 24 and 72 hr prior to toxin-LR challenge. The dose, 12.5 mg per mouse, was selected because it was similar to that of the hydrocortisone dose which was found in earlier studies to be protective against toxin-LR. More physiologic doses were not tested.

If impaired reticuloendothelial activity provided protection against toxin-LR, then heightening reticuloendothelial activity might increase sensitivity to the toxin. To test this, mice were treated with a saline suspension of zymosan A (Sigma Chem., St. Louis, MO) 1 mg/kg, iv, 2, 24, and 48 hr prior to toxin-LR challenge with 1.0, 0.9, or 0.8 x LD<sub>100</sub> and the time required for death to occur was recorded. To determine if the zymosan had indeed produced reticuloendothelial cell stimulation, the sheep red cell clearance test described above was used. Mice were injected with 1 mg of zymosan A and the spleen/liver uptake was evaluated at 2, 24, 48, 72, and 96 hr after injection.

6. Endothelial studies: Destruction of the sinusoidal architecture of the liver is a characteristic acute effect of

toxin-LR. Studies were devised to detect an early hepatic sinusoidal lesion and endothelial damage which might occur either locally or systemically. The procedures used included assessment of vascular permeability with  $^{125}\text{I}$ -albumin,  $^{51}\text{Cr}$ -labelled mouse red cells, and whole body autoradiography.

To quantitate temporal changes in tissue fluid distribution after toxin-LR, mouse albumin (Sigma Chem., St. Louis, MO) was labelled with  $^{125}\text{I}$  (Amersham Searle Corp., Arlington Hts., IL), then separated on a 1x25 cm Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ) column to a specific activity of 28  $\mu\text{Ci}/\mu\text{g}$ . Mice were injected at 0, 3, 8, 13, and 18 min post-toxin with  $^{125}\text{I}$ -albumin, then killed with ether 2 min post-isotope. For whole body autographs 6.6  $\mu\text{Ci}/75 \mu\text{l}$  was injected iv into 2 mice at each time period, and for distribution studies 0.4  $\mu\text{Ci}/100 \mu\text{l}$  was injected iv into 6 mice at each time period. Liver, lung, spleen, and kidneys were removed, weighed, and the  $^{125}\text{I}$  activity determined using a well-type scintillation detector. A 100  $\mu\text{l}$  sample of both blood and plasma was also counted. Data were expressed both as percent uptake per organ and percent uptake/g tissue normalized to a 25 g mouse.

To quantitate temporal changes in microvascular disruption after toxin administration, mouse red cells were labelled with  $^{51}\text{Cr}$  (Amersham Searle Corp., Arlington Hts, IL) in acid-citrate-dextrose solution and then rinsed 3 times with the same solution. Mice were injected at 0, 2, 7, 12, and 17 min post-toxin with the labelled red cells and then killed with ether 3 min post-isotope.

For distribution studies 0.31  $\mu\text{Ci}/100\mu\text{l}$  was injected iv into 5 mice at each time period. Liver, lung, spleen, and kidneys were removed, weighed, and  $^{51}\text{Cr}$  activity determined.

7. Chronic toxicity studies: Short-term and long-term studies of toxin-LR effects were initiated, the former to evaluate repeated sublethal exposure to the toxin and the latter to identify delayed effects of a single sublethal dose. Female Hale-Stoner mice of the same age were given intravenous injections of either toxin-LR or saline on Monday, Wednesday, and Friday for three weeks. The dose of toxin which was chosen was 50% of the  $\text{LD}_{100}$  dose used in acute toxicity studies. At the end of each week five animals of each group were sacrificed and the liver, spleen, lungs, and kidneys removed for weighing and histological examination. To see if any lesions which may have been produced healed or elicited a fibrotic reaction, some mice were observed a further 6 weeks without injections and then sacrificed.

Long-term pathogenicity was evaluated in both Hale-Stoner and Swiss-Webster mice. A single sublethal dose of toxin-LR, either 50% or 25% of the  $\text{LD}_{100}$  dose, was given intravenously. Weekly observations and monthly weighings were made for 6 mo. Mice were killed with ether, and organs were removed, weighed and fixed for histological examination.

8. Protective agents: Following the submission of the original contract proposal it was discovered that shellac, rather than

colloidal carbon, was the agent probably responsible for India ink-induced protection against toxin-LR. Further evaluation was made using India ink components kindly supplied by the Faber-Castell Co. (Newark, NJ). They included a 10% carbon suspension without shellac, a 39% solution of ammonium hydroxide-hydrolyzed gelatin, and a 23% solution of the ammonium salt of shellac without carbon. In addition, a principal component of shellac, aleuritic acid was obtained (Fluka Chem. Co., Hauppauge, NY). After solubilization of aleuritic acid in 1M  $\text{NH}_4\text{OH}$  or 1N  $\text{NaOH}$  to a final concentration of 40 mg/ml, pH was adjusted to 7.6 prior to injection. Bleached shellac, also furnished by Faber-Castell, was solubilized by heating at 60° C in 1N  $\text{NaOH}$  and adjusted to a pH of 7.6 providing a solution containing 80 mg of shellac per ml.

Subsequently Trypan blue was found to be protective against toxin-LR, and an evaluation of it and other diazo dyes was undertaken. Most of the dyes were purchased from Sigma Chemical Co. (St. Louis, MO). The general approach was to make 1% solutions of selected dyes in phosphate-buffered saline. A dose of 0.005 to 0.01 ml/gbw of dye solution was given ip either 1 hour, 1 day or 1 week prior to toxin-LR challenge. Not all the dyes tested were completely soluble in saline. Trypan red (Gurr, Biomedical Specialties, Santa Monica, CA), for example, was readily soluble in water. To determine the purity of Trypan blue and Evan's blue, thin layer chromatography was performed using 70% methanol in water containing 0.5 M ammonium acetate.

Multiple components were found in each dye. For example, Trypan blue from four different distributors had 5 to 7 distinct bands as determined by thin layer chromatography, some being detectable by fluorescence under uv light. The most effective Trypan blue formulation was distributed by Mallinckrodt, Inc. (St. Louis, MO), reagent number E125. Another group of dyes investigated in the same way were the triazines, also obtained from Sigma.

Earlier attempts to block the acute toxicity of toxin-LR by pretreating mice with another cyclic peptide toxin, phalloidin, were unsuccessful. It was hypothesized that, since the pathophysiologic effects and general structure of the two toxins were similar, an animal receiving the slower acting phalloidin followed by toxin-LR would succumb after an interval dictated by phalloidin toxicity if phalloidin and toxin-LR competed for the same receptor sites. To evaluate this hypothesis further, cyclosporine was obtained. This cyclic peptide is currently used for immunosuppression in man. Two formulations were used, the powdered compound (kindly provided by the Sandoz Co., East Hanover, NJ) and the intravenous preparation (Sandimmune®). The former was dissolved in Tween 80 (20%) and ethanol (80%) and injected daily by the subcutaneous route. Subsequently the intravenous formulation was used exclusively because of large local inflammatory reactions at sites of subcutaneous injection.

In previously reported studies (9) it had been found that hydrocortisone (12.5 mg/mouse, ip) injected up to 10 min after or 1 hr prior to toxin-LR would prevent lethality. Further

investigations were conducted using both hydrocortisone (0.5 or 1.0 mg/gbw, ip) and the long-acting potent synthetic glucocorticoid, dexamethasone (20 µg/gbw, ip or iv), the doses being given 240, 120, 60, and 30 min prior and 0.5, 5, 10, 15, and 20 min after toxin administration.

The availability of two other M. aeruginosa peptide toxins, toxin-RR and toxin-LA, permitted a comparison of their effects on mice that were given agents which protected against toxin-LR. In these studies an LD<sub>100</sub> dose of each toxin was given to mice that had been injected previously with a dose of a protective agent known to prevent toxin-LR lethality. In addition, the effectiveness of the toxins on neonatal (4 da) mice was tested.

## RESULTS AND DISCUSSION

1. Toxin production: Yields of toxin vary with the particular strain employed, cultural conditions, and phase of growth. We routinely obtain about 100 mg of a toxic peptide from a 1 kg lot of cells. Currently 300 mg of toxin-LR (cyanoginosin-LR), 50 mg of toxin-LA, and 50 mg of toxin-RR are available for experimental studies. Additional toxins are being prepared for comparative evaluation. Samples of toxin have been supplied to Dr. R. W. Warnemacher, Pathophysiology Division, USAMRRID, for mass spectroscopy evaluation and to Prof. A Meister, Department of Biochemistry, Cornell University Medical School, New York, NY,

for testing. Lyophilized cells of toxic strains of M aeruginosa have been sent to National Institutes of Health, National Cancer Institute for evaluation of antineoplastic components.

## 2. Pathophysiology:

a. Effect of different routes of administration on toxin-LR pathology. The gross morphologic changes following intraperitoneal, intravenous, or subcutaneous administration of toxin-LR were similar, although death occurred approximately 10 minutes sooner following intravenous injection. It was also observed that gross morphologic changes were not altered if toxin-LR was given by gavage or by intratracheal instillation. The lethal dose following gavage was much greater than the parenteral dose. Toxin-LR was administered by gastric tube to mice at 6 dose levels (Table 1). No mortality was seen until a dose 40 times the iv LD<sub>100</sub> dose was given. Gastrointestinal toxicity was increased more than three-fold by administering the toxin after withholding food for 16 hr. These data indicate that when food is not present in the stomach, toxin-LR absorption is increased and/or digestive breakdown is decreased. Death was just as rapid when toxin was given by gavage as when it was given ip if a sufficient dose was administered. The rapidity of death from intratracheal instillation approached that of parenteral toxin, and if twice the ip LD<sub>100</sub> dose was given by this route mortality was 100% (Table 1). This apparent decrease in toxicity may be due to a lack of toxin absorption and/or to metabolism of



toxin-LR in the lung.

b. Hepatic studies. Evidence that normal hepatocellular function was required for toxin-LR effects was evaluated by performing a partial hepatectomy on mice prior to toxin administration. Protection was afforded only if more than 75% of the liver was removed (Table 2).

The protection obtained by partial hepatectomy was due to either a deficiency of hepatocytes or by the small volume of remaining liver.  $^{51}\text{Cr}$ -labelled mouse erythrocytes, when injected 17 minutes after toxin-LR injection, localized primarily in the liver 3 minutes later (Fig. 3). Clearly, hepatic sequestration of blood and the resulting hypovolemia would have been minimized had only a small volume of liver been present.

A close temporal correlation was confirmed between the hepatic enlargement following toxin-LR injection and biochemical evidence of hepatocellular damage and thrombocytopenia (Fig. 4). Histologic changes may have begun prior to those events (Fig. 5-8). Possible disruption of sinusoidal architecture was present as early as 5-10 min after toxin administration. These changes were present primarily around the hepatic veins. Intrahepatic hemorrhage was apparent by 20 minutes. Lung tissue appeared normal at this time. By 30 min the liver hemorrhage was extensive and pulmonary emboli were present. A striking finding was a decrease in hepatic glycogen within 5 min after toxin injection. At 15 min post-injection no glycogen staining was

evident. The rapid depletion of glycogen may indicate loss of hepatocyte membrane integrity.

A number of biochemical parameters were tested serially in toxin-injected mice (Table 3). The significance of most of the observed changes have yet to be determined. But when a larger group of mice was studied, a statistically significant increase in serum lactate dehydrogenase activity was detectable as early as 5 min after toxin injection (Fig. 9). The role of toxin-LR as an hepatotoxin was studied using in vivo hepatocyte labelling with  $^{125}\text{I}$ IdUrd. It was observed that injection of 25% of the  $\text{LD}_{100}$  dose of toxin produced a release of the isotope even though gross morphologic changes in the liver were not apparent (data not shown). A sensitive assay for microanalysis for alanine aminotransferase was developed for work on mouse specimens. This assay can be performed on 10 $\mu$ l of sample and permits serial sampling of individual animals.

c. Endothelial studies. Studies using  $^{125}\text{I}$ -labelled mouse albumin indicated that permeability of the liver to albumin was relatively constant until 15-20 min after toxin-LR injection. At that point there was a sharp increase (Fig. 10). However, when % uptake of injected dose per g of tissue was calculated, the increase was not observed (Fig. 11). The lung permeability appeared to be biphasic. The reason for this is not clear. Whole body autoradiographic data indicated no significant change in the distribution of labelled albumin after toxin injection

(Fig. 12). Taken altogether, these experiments do not suggest that there is a preferential increase in vascular permeability to plasma components in the liver following toxin-LR injection.

Studies using  $^{51}\text{Cr}$ -labelled mouse erythrocytes to determine changes in vascular integrity revealed that the percent injected dose/g tissue (normalized to that of a 25 g mouse) increased in the liver between 15 and 20 min post-toxin (Fig.13). This correlates well with the histological and biochemical evidence of hepatocyte damage. There also was a steady increase in percent injected dose/g lung beginning as early as 5 min post-toxin. Therefore, despite the lack of histologic evidence of pulmonary congestion, stasis and/or extravasation of blood may occur in the lung shortly after toxin injection. In the spleen the quantity of labelled erythrocytes increased up to 10 min post-toxin and then declined, perhaps because the prominent hepatic sequestration of blood resulted in systemic hypovolemia.

d. Chronic toxicity studies. A clearly significant hepatic lesion was detected in mice receiving thrice-weekly injections of an  $\text{LD}_{50}$  dose of the toxin. The changes were apparent after 2 weeks and were more prominent at 3 weeks (Figs. 14, 15). The fully developed lesion surrounded the hepatic veins, leaving the portal areas apparently untouched. It consisted of hepatocytes in varying stages of degeneration and an infiltrate of mononuclear cells. Six weeks after stopping the injections some lesions were still present. No obvious fibrosis was occurring at that

time, at least as detectable by hematoxylin/eosin staining.

When sacrificed 6 mo after a sublethal dose of toxin-LR, test mice revealed no consistent pattern of organ damage different from control mice, as evaluated by gross tissue examination or by organ weight (Table 4). Histological sections remain to be interpreted.

### 3. Prophylaxis:

a. Protective agents. It was initially hypothesized that India ink protected mice against toxin-LR because of an effect on the reticuloendothelial system. Colloidal carbon in the ink was thought to be the active agent rather than the vehicle, which contained shellac and gelatin. However, when ink was centrifuged at 50,000xg for 4 hours, the protective effect was found to reside in the supernatant rather than the carbon pellet. Of the carbon, gelatin, and shellac formulations provided by the Faber-Castell Co., only the shellac was protective (Table 5). The critical role of shellac was confirmed when bleached shellac was obtained and solubilized in NaOH; it, too, was protective in a dose-dependent fashion (Table 6). A principal component of shellac, aleuritic acid, was ineffective in prophylaxis against toxin-LR. Shellac inhibited toxin-LR lethality and its characteristic hepatomegaly and severe thrombocytopenia as well.

The mechanism of shellac-induced protection was thought to involve macrophages because shellac is known to cause

reticuloendothelial blockade (20). It was observed that both the addition of shellac to plasma in vitro or its intravenous injection produced a turbidity to plasma. If shellac caused protection by producing an intravascular protein precipitation which then affected the reticuloendothelial system, serum protein abnormalities might have been produced. Serum protein electrophoresis indicated a modest decrease in total protein (data not presented), but no particular class of protein appeared to be preferentially decreased. Intravascular particulate matter could have resulted from intravascular coagulation. Coagulation studies revealed gross changes in the prothrombin, partial thromboplastin, and thrombin times following the intravenous administration of shellac (Table 7). Furthermore, the platelet count dropped immediately to one-half the normal value. Despite these findings it is unlikely that shellac-induced protection resides in these changes because mice which were defibrinated with Aqkistrodon rhodostoma venom (Sigma Chem. Co., St. Louis, MO) so as to be incoagulable were completely protected against toxin-LR after shellac administration, whereas the venom itself induced no protection.

Trypan blue, a diazo dye known to interfere with reticuloendothelial function and preferentially concentrate in macrophages (21), was found to block toxin-LR when given intravenously, but itself was very toxic in saline solution. Subsequently the intraperitoneal route was used, and with larger doses of the dye protection against the toxin was sustained for

up to 3 weeks. Later it was discovered that ready solubility was obtainable in water so that larger doses could be given intravenously. It is probable that small particles of insoluble dye (or dye contaminants) were the source of acute toxicity noted in earlier experiments using Trypan blue given in saline.

A partial list of dyes tested (Table 8) includes Trypan red. This remarkable compound was found to provide protection against toxin-LR for up to 3 months after a single intraperitoneal injection. Duration of the protection was dose-dependent (Table 9). In contrast to Trypan blue, no liver damage was detectable after its administration, clearly showing that hepatocyte dysfunction was not required for blocking toxin-LR (Table 10). Concentrated deposits of Trypan red could be detected in the bone marrow of treated mice, and counterstaining with Prussian blue indicated that at least some of the cells concentrating Trypan red were macrophages (Fig. 15). A colorless diazo derivative, suramin (Mobay Chem. Corp., New York NY) is currently used in the treatment of trypanosomiasis in humans. It was found to be minimally protective against toxin-LR in mice even when given in daily doses (0.01 ml/gbw of a 0.2% solution) to the point of toxicity of the drug.

Triazine dyes were tested because it had been noted that the plasma of Trypan blue-treated animals remained blue-colored for many days. Also, diazo dyes effective for protection tended to stain mice, whereas ineffective ones did not. This suggested that binding to plasma proteins, primarily albumin, might somehow

be involved in protection. Diphenylhydantoin, which binds extensively to plasma proteins, was ineffective in prophylaxis at a dose of 50 mg/Kg (data not shown). However, triazine dyes also bind to proteins, and at least one such compound, Reactive Red 120, was found to be highly protective (Table 8). It may be pertinent that this dye contains a diazo linkage.

Cyclosporine is a cyclic peptide used as an immunosuppressive agent in human organ transplantation (22). Cyclosporine blocked toxin-LR lethality when given either subcutaneously or intravenously, and protection was apparent at a lower dose by the latter route (Table 11). As with Trypan red, duration of protection was dose-dependent as well as being present within two minutes of injection. The vehicle for the intravenous formulation of cyclosporine (Sandimmune iv<sup>®</sup>) was kindly provided by the Sandoz Company. It had no protective effect.

All of the protective drugs discovered, except for the triazines, were tested for their ability to prevent death when given after toxin-LR injection. It was known from earlier studies that hydrocortisone was "therapeutic" when given as late as 15 minutes after the toxin. Of the other drugs, none were clearly therapeutic (Table 12), although Trypan red was protective if given 10 minutes after the toxin. However, as parenteral toxin administration was used in all experiments, the relevance of the findings to clinical exposure is not known.

Despite the apparent similarity of the lethal dose, time to

death, and gross organ pathology produced by the three toxins (-LR, -RR, -LA), there was a striking difference in their inhibition by the protective agents (Table 13). Toxin-LA, in which leucine and alanine are present, was blocked only by shellac. Of particular interest is that newborn mice and CCl<sub>4</sub>-treated mice were not protected against toxin-LA. These new observations are of prime importance to an understanding of both the mechanism of action and the metabolism of this group of cyclic peptide toxins.

b. Reticuloendothelial studies. The ratio of the spleen to liver uptake of radiolabelled sheep red cells in control mice, which was assessed in all assays of in vivo reticuloendothelial function, averaged  $0.08 \pm 0.03$  (n=55). A summary table of the results of assays following the administration of many dyes (Table 8) includes the degree of reticuloendothelial blockade produced by "positive" control mice that had received another agent known to interfere with macrophage function, dextran sulfate 500. This compound was ineffective at blocking toxin-LR. Selected agents which had been discovered to inhibit the toxin were studied longitudinally (Fig. 17). There was a rough correlation between the times when an agent was protective and the presence of some degree of reticuloendothelial blockade. Trypan red, however, was a notable exception in that the ratio of spleen to liver uptake of labelled cells did not vary even though



the level of protection against the toxin did vary. Nevertheless, Trypan red was noted to be concentrated in bone marrow macrophages. Taken altogether, there is a suggestion that the reticuloendothelial system is involved in toxin activation or processing, but the dextran sulfate 500 and Trypan red data indicate that caution must be observed in attributing too much importance to such an concept at this time.

No significant sex-related difference in susceptibility to toxin-LR was found in Hale-Stoner mice (data not shown). However, in preliminary studies, when soluble estrogens (up to 12.5 mg/mouse) were given to either male or female mice, protection was induced that lasted up to 3 days (data not shown). While only limited observations of this phenomenon were made, pharmacologic doses were clearly required. In contrast, dexamethasone, a glucocorticoid which is approximately 25 times more potent than hydrocortisone, was found to be ineffective in prophylaxis against toxin-LR (Table 14).

Zymosan is a reticuloendothelial stimulant and produces reticuloendothelial cell hyperplasia (23). Despite this, no shortening of survival or increased susceptibility to lower toxin-LR doses was observed in zymosan-pretreated mice (Table 15). It may be pertinent that zymosan produced a moderate degree of reticuloendothelial blockade, as indicated by sheep red cell clearance studies (Table 16). Perhaps the inhibition of the macrophage clearance activity by zymosan overrode any stimulation of other macrophage actions by zymosan.

## CONCLUSIONS

The original contract proposal for the present studies centered on investigation of the pathophysiology of a well-characterized M. aeruginosa cyclic peptide toxin designated toxin-LR and on prophylaxis against it. At this point the critical role of the liver in both the pathogenicity of and protection against toxin-LR in mice seems established. The particular cell type responsible for these effects, however, is not yet known.

The protection against toxin-LR which is known to exist after treatment of mice with the hepatotoxin, carbon tetrachloride, is also evident after extensive hepatectomy. The mechanism of the protection, however, cannot be unequivocally explained by hepatocyte damage. Studies of sheep red cell clearance reveal that there is impaired reticuloendothelial function in addition to hepatocyte damage in mice receiving carbon tetrachloride. Furthermore, a characteristic toxin-LR finding, a large, hemorrhagic liver which may contain up to one-half of the circulating blood volume (based on increase in liver weight; see reference 6) cannot occur in extensively hepatectomized animals. Thus, the role of the hepatocyte in producing the pathophysiology of acute toxin-LR toxicity remains to be determined. In addition, while there is evidence of acute

hepatocyte injury following the administration of toxin-LR, much of the observed alanine aminotransferase release following an LD<sub>100</sub> dose of toxin-LR might be due to severe intrahepatic hemorrhage caused by the toxin. It is not certain, therefore, that the hepatocyte is an important target of the acute toxin-LR toxicity.

Studies of the various prophylactic agents so far discovered (carbon tetrachloride, hydrocortisone, shellac, diazo dyes, triazine dyes, and cyclosporine) are compatible with the Kupffer cell being the common denominator in their protection. With the exception of Trypan red, some degree of impaired clearance of sheep red cells has been found following the administration of each of the protective agents. This is not to say that the particle clearance function of the macrophage is the actual mechanism through which the protective agents act. Dextran sulfate markedly impaired sheep red cell clearance in mice, but induced no protection against toxin-LR. If macrophages are indeed involved, it is more likely that impaired particle clearance is a marker for a deranged macrophage and that other activities such as pinocytosis or lysosomal function are more intimately involved in protection.

Since sinusoidal disruption is a major pathophysiologic event in toxin-LR lethality, it is possible that endothelial cells or structural integrity of the endothelial lining of sinusoids is a target of toxicity. Morphological studies suggest sinusoidal separation may precede overt intrahepatic hemorrhage.

However, studies using radiolabelled albumin and autoradiography, have not confirmed this hypothesis, and so far no definite early endothelial lesion has been uncovered. Perhaps such a lesion, if it exists, develops rapidly.

The most efficient protective agents yet found are Trypan red and cyclosporine in that protective doses, in  $\mu\text{g/gbw}$ , approach that of the  $\text{LD}_{100}$  dose of toxin-LR. Trypan red is remarkable for the duration of its protection in mice, which approaches 3 months and is equivalent to 10-15% of the life span. Like Trypan blue, Trypan red is taken up, at least in part, by bone marrow macrophages, and it is therefore likely that Kupffer cells will also be found to concentrate the dye. Cyclosporine protection may be an especially useful discovery since it is already in wide use in humans. Cyclosporine is thought to have minimal effects on macrophages. The low doses of Trypan red and cyclosporine that are required for protection suggest some specificity in mechanism, in contrast, for example, to the large doses of hydrocortisone which are needed for protection.

There is, nevertheless, good reason to study further the effects of the general class of steroidal agents in blocking cyclic peptide toxicity. The mechanism of the protection afforded by hydrocortisone does not appear to be modulated through its glucocorticoid effects. Dexamethasone, a glucocorticoid which is approximately 25 time more potent, was found to be ineffective in prophylaxis even though the dose, in  $\text{mg/gbw}$ , was only one-half that of hydrocortisone. Possible

explanations for the effectiveness of hydrocortisone and conjugated estrogens include a nonspecific membrane-modifying effect of high doses of the steroidal lipids and a mineralocorticoid effect of hydrocortisone, which is known to be minimal for dexamethasone.

The chronic toxicity of repeated sublethal doses of toxin-LR has received limited study, but a definite lesion has been identified. It is likely that direct hepatocyte toxicity is involved in this phenomenon because degenerating hepatocytes in the absence of vascular disruption are histologically apparent. Delayed effects of a single toxin-LR challenge are still being evaluated histologically.

The recent availability of two more purified cyclic peptides of M. aeruginosa, toxin-LA and toxin-RR, has provided a new opportunity to investigate toxin pathophysiology and prophylaxis. Although gross anatomical changes and acute thrombocytopenia are similar among the three toxins, the different patterns of protection by the prophylactic agents so far discovered strongly suggest that a single amino acid substitution may critically alter toxin metabolism. Specifically, the lethality of toxin-LA has been found to be unaffected by most of the protective agents. This may lead to an understanding of the molecular basis of action of toxin-LR and related cyclic peptide toxins. Tritiated toxins, which are in an early stage of development, would be useful in this regard.

In conclusion, the first year of study of the

pathophysiology and prophylaxis of M. aeruginosa cyclic peptide toxins has resulted in some exciting and unexpected findings. In addition, it has already been found that many of the observations on toxin-LR prophylaxis also apply to two cyclic peptide toxins of Amanita phalloides,  $\alpha$ -amanitin and phalloidin. A sample of phalloidin, kindly provided by Prof. T. Wieland of the Max Planck Institute, was studied in addition to the Sigma products used for most of the mushroom toxin studies. The effectiveness of the protective agents against Prof. Wieland's phalloidin is summarized in Table 17.

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Table 1: Acute toxicity of toxin-LR following gastrointestinal and intratracheal administration\*.

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Route of

administration      Toxin dose ( $\mu\text{g/gbw}$ )      Mortality\*\*

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Gavage:	0.4	0/7
	1.0	0/2
	2.0	0/6
	4.0	1/8
	5.0	4/17
	5.0***	14/18

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Intratracheal:	0.1	0/16
	0.15	4/8
	0.2	16/16

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\* LD<sub>50</sub> for toxin-LR given parenterally is 0.1  $\mu\text{g/gbw}$ .

\*\* Death occurring in less than 3 hr.

\*\*\* Fasted for 16 hr prior to toxin administration.

Table 2: Relationship between estimated percent liver removal\* and time to death after toxin-LR administration (0.1 µg/gbw, iv).

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Body weight (g)	Estimated percent liver removal	Time to death after toxin-LR
32.5	30	75 min
32.6	40	36 min
32.7	40	56 min
33.0	65	59 min
31.4	65	58 min
31.4	65	44 min
32.2	70	75 min
33.4	70	59 min
28.1	75	>16 hr
30.7	75	>24 hr
30.9	75	>16 hr
29.7	80	>16 hr
27.8	80	>48 hr

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\* Partial hepatectomy performed 24 hr prior to toxin-LR challenge.

Table 3: Serum chemistry values (mean  $\pm$  S.D.) following toxin-LR injection (0.1  $\mu$ g/gbw, iv) in mice (5-10 per group).

Group	Alkaline	Aspartate	Alanine	Glucose
	phosphatase	aminotransferase	aminotransferase	
Group	(mg/dl)	(U/l)	(U/l)	(mg/dl)
Control	81 $\pm$ 8	373 $\pm$ 126	75 $\pm$ 13	184 $\pm$ 15
5 min	85 $\pm$ 10	390 $\pm$ 194	73 $\pm$ 29	208 $\pm$ 8
10 min	77 $\pm$ 9	311 $\pm$ 34	64 $\pm$ 21	213 $\pm$ 37
15 min	100 $\pm$ 11	300 $\pm$ 67	119 $\pm$ 41	208 $\pm$ 7
20 min	78 $\pm$ 8	785 $\pm$ 315	339 $\pm$ 185	208 $\pm$ 26
25 min	81 $\pm$ 6	1006 $\pm$ 244*	893 $\pm$ 257***	271 $\pm$ 12***
30 min	76 $\pm$ 6	1842 $\pm$ 369**	1446 $\pm$ 245***	311 $\pm$ 35***

Group	Triglycerides	Albumin	Total	Lactate
			protein	dehydrogenase
Group	(mg/dl)	(gm/dl)	(gm/dl)	(U/l)
Control	170 $\pm$ 12	2.7 $\pm$ 0.1	5.1 $\pm$ 0.1	1518 $\pm$ 355
5 min	164 $\pm$ 7	2.8 $\pm$ 0.1	5.1 $\pm$ 0.1	1220 $\pm$ 350
10 min	142 $\pm$ 2	2.8 $\pm$ 0.1	5.0 $\pm$ 0.1	1122 $\pm$ 63
15 min	94 $\pm$ 18***	2.8 $\pm$ 0.1	5.5 $\pm$ 0.1	1268 $\pm$ 160
20 min	91 $\pm$ 15***	2.7 $\pm$ 0.1	5.0 $\pm$ 0.1	2938 $\pm$ 447*
25 min	124 $\pm$ 25	2.6 $\pm$ 0.1	4.7 $\pm$ 0.2	2679 $\pm$ 555
30 min	114 $\pm$ 15**	2.5 $\pm$ 0.1*	4.3 $\pm$ 0.1***	3916 $\pm$ 469***

\* p < 0.05; \*\* p < 0.02; \*\*\* p < 0.01.

Table 4: Long-term\* effects of a single sublethal iv dose of toxin-LR on the organ weights of female mice of two strains\*\*.

Hale-Stoner mice:	Control	0.25 LD <sub>100</sub> ***	0.50 LD <sub>100</sub>
Body weight (g)	35.8±1.0	33.4±1.0	35.6±1.5
Liver weight (g)	1.72±0.09	1.84±0.06	1.66±0.07
Lung weight (mg)	185±34	189±6	184±7
Spleen weight (mg)	243±16	263±23	258±32
Kidney weight (mg)	438±9	449±15	417±13
Heart weight (mg)	144±3	147±5	135±5
Thymus weight (mg)	118±10	114±7	115±12
Swiss-Webster mice:	Control	0.25 LD <sub>100</sub>	0.50 LD <sub>100</sub>
Body weight (g)	39.2±1.0	37.6±0.8	37.6±0.3
Liver weight (g)	1.70±0.04	1.70±0.04	1.57±0.04****
Lung weight (mg)	213±10	202±5	213±5
Spleen weight (mg)	174±10	190±20	166±10
Kidney weight (mg)	397±10	406±10	385±10
Heart weight (mg)	136±30	134±20	144±3
Thymus weight (mg)	88±5	87±10	75±5

\* Hale-Stoner mice were killed 176 da after toxin injection;

Swiss-Webster mice were killed after 161 da.

\*\* Each group contained 20 mice.

\*\*\* LD<sub>100</sub> dose of toxin-LR = 0.1 µg/gbw.

\*\*\*\* Significantly different from control mice (p < 0.05) by t-test.

Table 5: Effectiveness of India ink components in preventing the lethality of toxin-LR (0.1 ug/gbw, ip) in mice\*.

Component	Dose (ml/mouse, iv)	Protective effect**
Carbon suspension	0.20	-
	0.10	-
	0.05	-
Gelatin solution	0.20	-
	0.10	-
	0.05	-
Shellac solution	0.01	+
	0.005	+
	0.0025	-

\* Faber-Castell Co., Newark, NJ, kindly provided components of Waterproof Drawing Ink, No. 4415. They included a 10% carbon suspension, a 39% solution of ammonium hydroxide-hydrolyzed gelatin, and a 23 % solution of the ammonium salt of shellac. The volume of India ink found to be protective was 0.05 ml/mouse.

\*\*Toxin-LR was injected 30 minutes after administration of each of the components.

Table 6: Duration of protection against toxin-LR (0.1  $\mu$ g/gbw, iv) after administration of solubilized shellac\*.

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Dose of shellac (mg)	Interval between treatment and toxin (hr)	Mortality
4	0.75	0/15
8	0.75	0/5
4	2	0/5
8	2	0/5
4	24	0/5
8	24	0/5
4	48	0/5
8	48	0/5
4	120	1/3
8	120	0/4
4	168 (7 days)	8/10
8	168	9/10
4	264 (11 days)	10/10
8	264	10/10

\* "Bleached shellac", Faber-Castell; Na salt, pH 7.6, given iv.

Table 7: Hemostasis studies\* one hour following intravenous injection of shellac (0.01 ml of 23% solution of the ammonium salt, Faber-Castell).

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Partial				
Test	Prothrombin	Thromboplastin	Thrombin	Platelet
<u>substance</u>	<u>time</u>	<u>time</u>	<u>time</u>	<u>countx10<sup>3</sup></u>
Saline	11 sec	37 sec	30 sec	1224±113
Shellac	28 sec	>2 min	>2 min	524±48

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\* Mean values of coagulation tests are from 4 pools of sera.

Platelet counts (±S.D.) are from 5-10 mice per group.

Table 8: Protection against toxin-LR and sheep red cell (SRBC) clearance following ip injection of diazo and triazine dyes.

		Time of SRBC	Spleen/liver	Inhibits
Treatment	Dose	injection*	radioactivity	toxin-LR
Saline			0.05 ± 0.01	No
Dextran SO <sub>4</sub>	750 µg	2 hr	0.13	No
		3 da	1.21	No
Trypan blue	20 mg/kg	3 da	0.76	Yes
Trypan red	20 mg/kg	1 da	0.09 ± 0.07	Yes
		3 da	0.09 ± 0.09	Yes
Evan's blue	5 mg/kg	1 da	0.07 ± 0.02	Yes
		3 da	0.12 ± 0.03	Partial
Vital red	20 mg/kg	1 da	0.16 ± 0.13	Yes
		3 da	0.12 ± 0.02	Yes
Reactive red 120	20 mg/kg	1 da	0.15 ± 0.40	Yes
		3 da	0.07 ± 0.02	Yes
Reactive blue 2	20 mg/kg	1 da	0.07 ± 0.04	No
		3 da	0.05 ± 0.20	No

\* Prior to toxin-LR injection.



Table 9: Correlation between dose of Trypan red and the duration of protection against toxin-LR, 0.1 ug/gbw, iv.

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Time after	Dose of Trypan red (mg/kg, iv)							
Trypan red	100	50	25	12.5	6.0	3.0	0.75	0.28
1 hr	0*	0	0	0	0	0	33	100
1-2 da	0	0	0	0	33	100	100	100
1 wk	0	0	50	100	100	100	100	100
1 mo	0	***	-	-	-	-	-	-

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\* Fractional mortality, percent. The number of animals in each group ranged from 3 to 10.

\*\* Not done.

Table 10: Serum alanine aminotransferase activity following ip injection of Trypan dyes or carbon tetrachloride.

Time after injection	Trypan blue (0.1 mg/qbw)	Trypan red (0.2 mg/qbw)	CCl <sub>4</sub> (pos. controls) (0.05 ml/mouse, sc)
6 hr	44 ± 8*	39 ± 11	39 ± 10
2 da		55 ± 6	6827 ± 1019
4 da	> 3000		
6 da	397 ± 229	41 ± 11	
14 da	51 ± 19	36 ± 8	
21 da		40 ± 10	
28 da		41 ± 20	

\* IU per liter ± S.D. Five mice per group. Addition of the dyes to control mouse plasma having either normal or elevated enzyme levels did not affect the enzyme activity readings.

Table 11: Protective effects of cyclosporine against toxin-LR  
(0.1  $\mu$ g/gbw, i.v.).

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Mortality				
Cyclosporine Prep. A*	Prep. B**	Prep. B	Prep. B	
Dose (mg)	1 hr***	24 hr	48 hr	72 hr
1.250	0/10	0/18	4/8	5/8
0.625	0/10	6/20		
0.313	0/10			
0.250		12/20		
0.200	0/10			
0.125		10/10		
0.100	1/10			
0.050	9/10			
0.025		10/10		
0.013	20/20	19/20	16/16	6/7

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\* Preparation A is Sandimmune i.v.<sup>®</sup> (cyclosporine 50 mg, polymethylated castor oil 650 mg, alcohol 32.9% by volume, 0.9% NaCl added to make 8 ml) injected intravenously.

\*\* Preparation B is cyclosporine 25 mg/ml in 20% Tween 80, 80% ethanol, injected subcutaneously.

\*\*\* Time of administration prior to toxin-LR.

Table 12: Effectiveness of prophylactic agents in blocking toxin-LR lethality when given after toxin-LR (0.1 ug/gbw, iv)

Protective		Minutes after toxin-LR injection							
agent*	Dose	0	1	2	3	4	5	10	15
Shellac	0.05 ml/mouse, iv	+	+	-	-	-	-	-	-
Hydrocortisone	12.5 mg/mouse, ip	+					+	+	+/-
Trypan red	1 mg/kg, iv	+					+	+	-
Cyclosporine	0.2 mg/kg, iv	+	+	+	-	-	-	-	

(+) = effective; (-) = ineffective.

Table 13: Summary of observations on the effectiveness of protective agents in blocking the lethality of three cyclic peptide toxins of M. aeruginosa.

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Protective status**	Test Agent*:		
	Toxin-LR	Toxin-RR	Toxin-LA
CCl <sub>4</sub>	yes***	yes	no
Trypan red	yes	yes	no
Cyclosporine	yes	yes	no
Shellac	yes	yes	yes
Newborn	yes	partial	no

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\* The toxin dose, given ip, is the LD<sub>100</sub> described in the Results and Discussion section.

\*\* Timing and/or dose which is known to protect against toxin-LR.

\*\*\* Effectiveness in protection.

Table 14: Comparison of hydrocortisone and dexamethasone in protecting mice against toxin-LR challenge (0.1  $\mu$ g/gbw, iv).

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Steroid	Dose (ip)	Minutes prior to toxin-LR			
		240	120	60	30
Hydrocortisone	0.50 mg/gbw	6/9*	1/14	1/12	1/12
Dexamethasone	0.02 mg/gbw	5/5	4/5	5/5	4/5
Dexamethasone	0.20 mg/gbw	10/10	15/15	12/12	9/10

---

\*Fractional mortality.

Table 15: Effect of prior injection of zymosan (1 mg/qbw, ip) on sensitivity of mice to toxin-LR.

Toxin-LR dose (iv) (X LD <sub>50</sub> dose)	Time to death [mean $\pm$ S.E. (survivors)]			
	Controls	2 hr*	24 hr	48 hr
1.0	54 $\pm$ 5	57 $\pm$ 31 (1)	58 $\pm$ 13	44 $\pm$ 10
0.9	64 $\pm$ 5	62 $\pm$ 14 (2)	51 $\pm$ 10 (1)	50 $\pm$ 7
0.8	74 $\pm$ 9	72 $\pm$ 9 (5)	113 $\pm$ 21	99 $\pm$ 24

\* Time toxin was injected after zymosan.

Table 16: Effect of ip injection of zymosan (1 mg/gbw) on sheep red cell clearance in mice.

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<u>Test group*</u>	<u>Spleen/liver radioactivity <math>\pm</math> S.D.</u>	<u>p value**</u>
Control	0.022 $\pm$ 0.003	
2 hr	0.092 $\pm$ 0.020	< 0.01
24 hr	0.134 $\pm$ 0.027	< 0.005
48 hr	0.082 $\pm$ 0.014	< 0.005
72 hr	0.045 $\pm$ 0.009	< 0.05
96 hr	0.038 $\pm$ 0.005	< 0.05

---

\* 5 mice per group.

\*\* Compared to control, student t-test.



Table 17. Protective agents and their duration of protection of Swiss mice against a lethal dose of phalloidin.

Agent (dose)	Times after administration of protective agent that phalloidin was injected			
	1 hr	1 da	1 wk	1 mo
Shellac (0.05 ml, iv)	0/10	0/10	8/10	
Trypan red (0.1 mg/gbw, iv)	0/10	0/10	0/10	
Hydrocortisone (12.5 mg, ip)	0/10	7/10		
Reactive Red 120 (0.2 mg/gbw, ip)	0/10	0/10	0/10	
Cyclosporine (0. mg, iv)	0/10	10/10		
Isotonic saline (0.01 ml/gbw, iv)	10/10	10/10	10/10	

- Phalloidin for this experiment was generously provided by Prof. T. Wieland of the Max Planck Institute. The challenge dose of phalloidin, 3  $\mu$ /gbw, was given ip at the specified times after administration of the protective agents.

## FIGURE LEGENDS

Figure 1: The arrows indicate an occlusive pulmonary vascular lesion in the lung of a mouse 30 min after injection of a lethal dose of toxin-LR (0.1  $\mu$ g/gbw). The other transected vessel contains erythrocytes. Phosphotungstic acid-hematoxylin stain, 400 X.

Figure 2: Preferential concentration of  $^{51}$ Chromium-labelled platelets in the liver following the injection of a mouse with a lethal dose of toxin-LR (0.1  $\mu$ g/gbw). Open bars = control mice; hatched bars = mice in which intravascular coagulation was induced by injection of a mixture of epinephrine and collagen; cross-hatched bars = toxin-LR-injected mice.

Figure 3: Organ uptake of  $^{51}$ chromium-labelled mouse erythrocytes at specified times after injection of a lethal dose of toxin-LR (0.1  $\mu$ g/gbw) into mice. The liver is the primary site of localization 20 min after toxin injection.

Figure 4: Temporal association of onset of thrombocytopenia, hepatocyte injury, and liver enlargement following the injection of a lethal dose of toxin-LR (0.1  $\mu$ g/gbw) into mice at time zero. Units: platelet count  $\times 10^{-3}/\mu$ l; liver weight as percent of body weight; alanine aminotransferase in IU/l  $\times 10^3$ .

Figure 5: Normal liver, with an hepatic vein indicated by the arrow. Hematoxylin/eosin stain, 100 X.

Figure 6: Separation of hepatocytes, perhaps as a result of sinusoidal (endothelial) damage or increase in permeability, may be an early hepatic lesion produced by injection of mice with a lethal dose (0.1  $\mu$ g/gbw) of toxin-LR. These changes, which occurred around the hepatic veins (see arrow) but not around the portal triads, were apparent 5-10 min after toxin administration. Hematoxylin/eosin stain, 400 X.

Figure 7: As the toxin-LR damage progresses, areas of hemorrhage into the hepatic parenchyma are apparent near the hepatic veins (see arrow). Hematoxylin/eosin stain, 100 X.

Figure 8: The final stage of toxin-LR-induced hepatic damage is characterized by a large, friable liver and nearly total disruption of liver architecture. Hematoxylin/eosin stain, 100 X.

Figure 9: Serum enzyme changes following a lethal dose of toxin-LR (0.1  $\mu$ g/gbw, iv) in mice. The elevation in serum lactate dehydrogenase activity 5 min after toxin injection is statistically significant, a finding which was not noted in the results presented in Table 3, where fewer animals were used at each time point.

Figure 10: The distribution of  $^{125}$ I-labelled mouse albumin following the injection of mice with a lethal dose (0.1  $\mu$ g/gbw)

of toxin-LR. The uptake is graphed as percent of injected dose. The increase of the label in the liver occurs at a time when liver enlargement is occurring (see Fig. 4).

Figure 11: The distribution of  $^{125}\text{I}$ -labelled mouse albumin following the injection of mice with a lethal dose ( $0.1 \mu\text{g/gbw}$ ) of toxin-LR, with uptake being represented as percent of injected dose/g tissue, normalized to that of a 25 g mouse. In contrast to the distribution noted in Fig. 9, the uptake of the label in the liver is not increased. This finding does not suggest that hepatic sinusoidal disruption produced by the toxin is associated with an early endothelial lesion which causes an increase in hepatic sinusoidal permeability.

Figure 12: Whole-body autoradiography of mice injected with  $^{125}\text{I}$ -labelled mouse albumin following injection of a lethal dose ( $0.1 \mu\text{g/gbw}$ ) of toxin-LR does not reveal a localized increase in the label in any organ when compared to control mice receiving no toxin. The mice were killed at the specified times after toxin-LR injection.

Figure 13:  $^{51}\text{Cr}$ -labelled mouse erythrocytes, when injected into mice following a lethal dose ( $0.1 \mu\text{g/gbw}$ ) of toxin-LR, reveal varying tissue distributions when graphed as percent of injected dose per g/tissue, normalized to a 25 g mouse. Notably, there is a progressive increase in the label in the lung and a transient increase in the label in the spleen.

Figure 14: Hepatic lesions (see arrow) develop after two weeks of thrice-weekly injections of an LD<sub>50</sub> dose of toxin-LR. Hepatocyte degeneration and round cell infiltration are present. Sections are stained with hematoxylin/eosin; 100 X.

Figure 15: After three weeks of thrice-weekly injections of an LD<sub>50</sub> dose of toxin-LR the lesions shown in Fig. 14 have become more extensive (see arrows) and more numerous. The round cell infiltration and hepatocyte necrosis occur primarily around the hepatic veins, although there is some hepatocyte necrosis scattered throughout the liver parenchyma. Hematoxylin/eosin stain, 100 X.

Figure 16: The ability of macrophages to take up Trypan red is shown in the cell indicated by the arrow, which contains both blue-green iron granules (stained by the Prussian blue reaction) and red dye. The marrow is otherwise unstained; 400X.

Figure 17: Assays of reticuloendothelial cell function in mice following administration of agents found to prevent lethality of toxin-LR were performed using <sup>51</sup>Cr-labelled sheep erythrocytes and determining their uptake in the liver and spleen at the indicated times after injection of the protective agents. The result of each assay is expressed as a ratio of the spleen to liver uptake. The horizontal dashed lines indicate the normal range ( $\pm$  SD) in control mice. The ordinate scale represents 1)

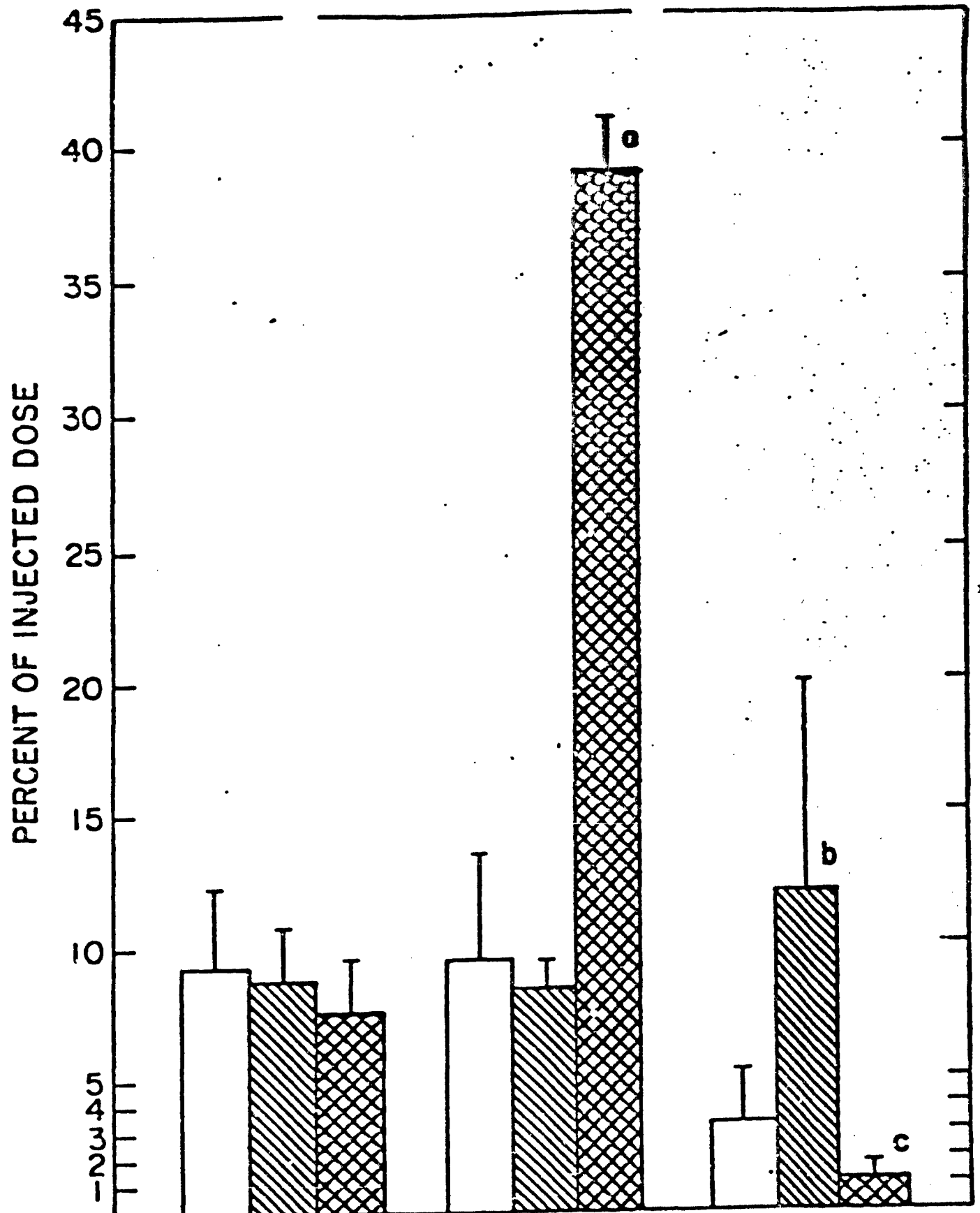
the ratio of the test mice divided by the ratio of concurrently run control mice (open bars), and 2) the fractional survival ( $\times 10^{-4}$ ) of mice given the specified protective agent and then challenged with a lethal dose (0.1  $\mu$ g/gbw) of toxin-LR (solid bars). The time the toxin was given after the administration of the protective agents is indicated on the abscissa. Doses and injection routes: hydrocortisone - 12.5 mg/mouse, iv; shellac - 0.01 ml of a 27% aqueous solution of the ammonium salt per mouse, iv; Trypan blue - 0.1 mg/gbw, ip; CCl<sub>4</sub> - 0.05 ml, sc; triazine dyes - 0.1 mg/gbw, ip; Trypan red - 0.2 mg/gbw, ip. The individual graphs represent the results of two or more experiments. The numbers of mice per assay group ranged from 4 to 10. The numbers of mice per group for toxicity testing ranged from 5 to 10.



FIGURE 1.

FIGURE 2

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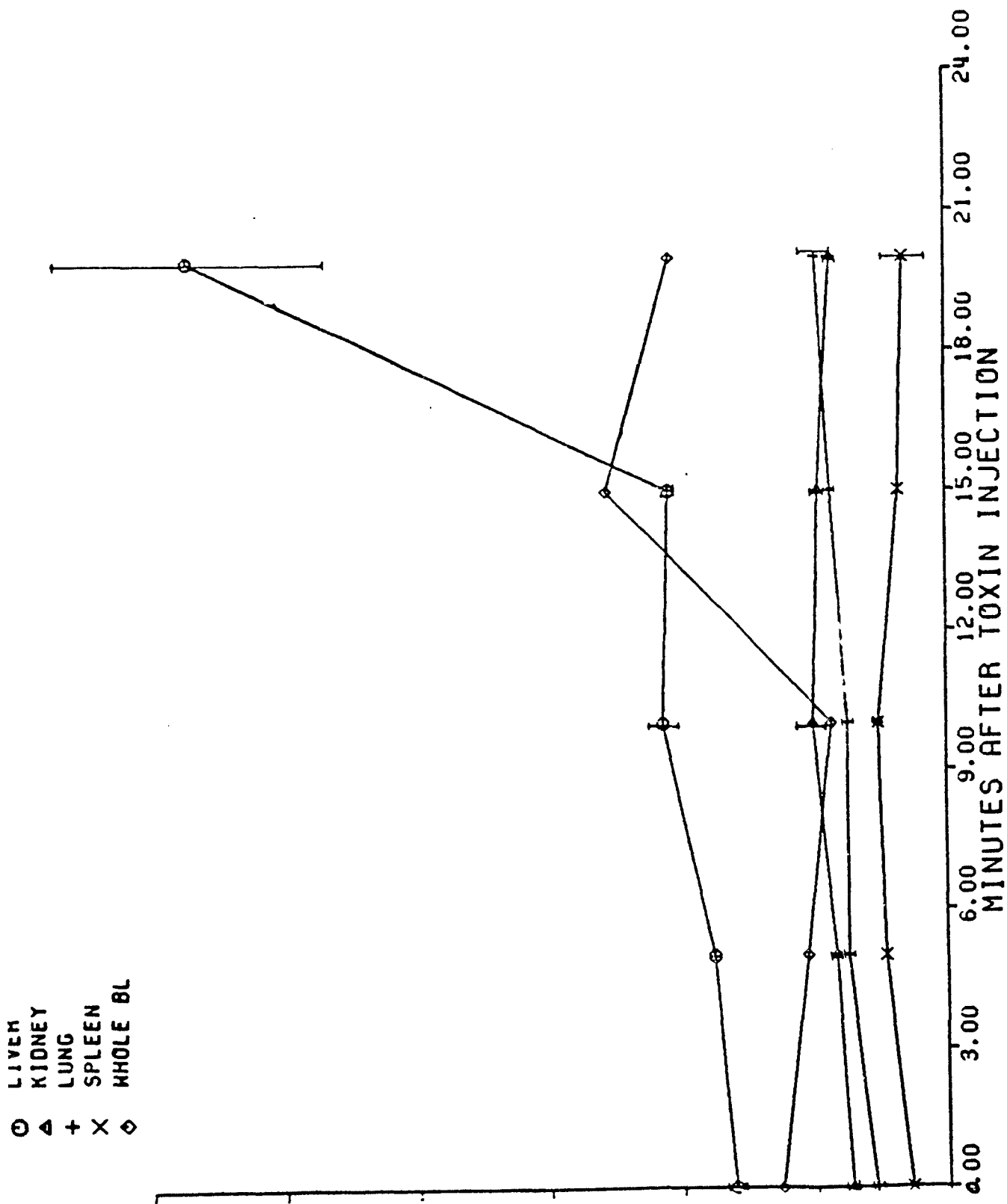


FIGURE 3 . UPTAKE OF MRBC-CRS1 AFTER TOXIN-LR

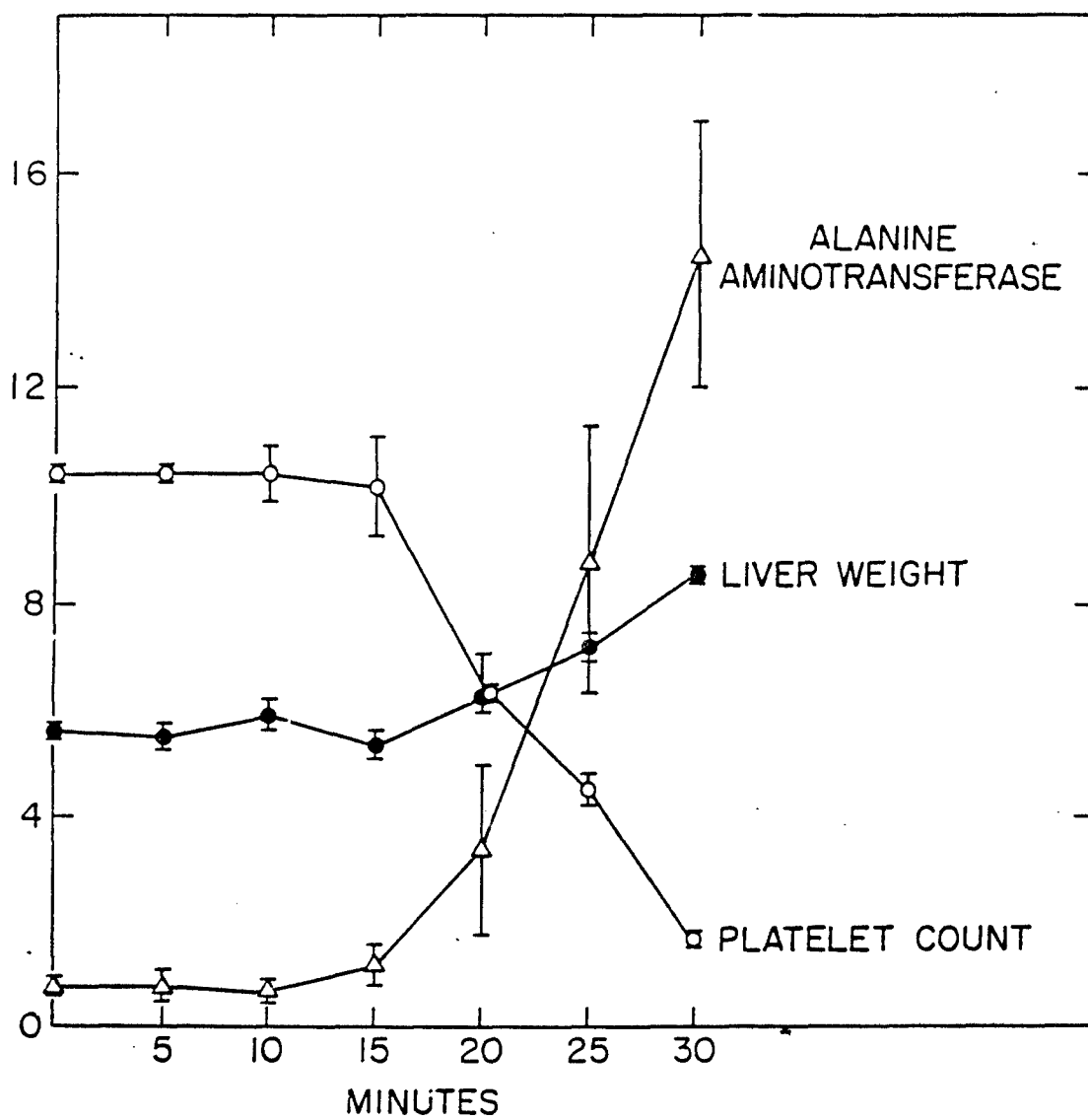


FIGURE 4



FIGURE 5



FIGURE 6

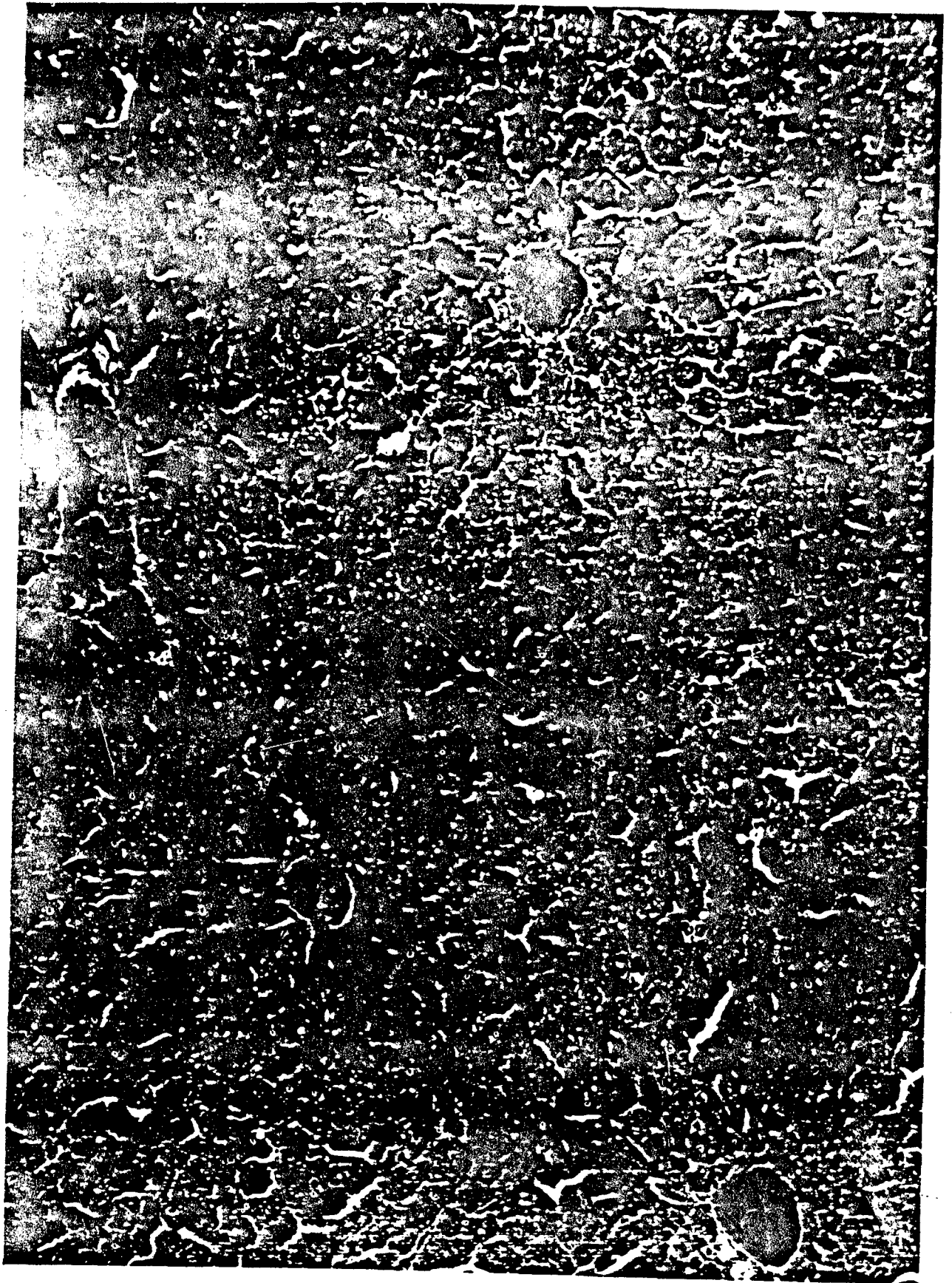


FIGURE 7



FIGURE 8



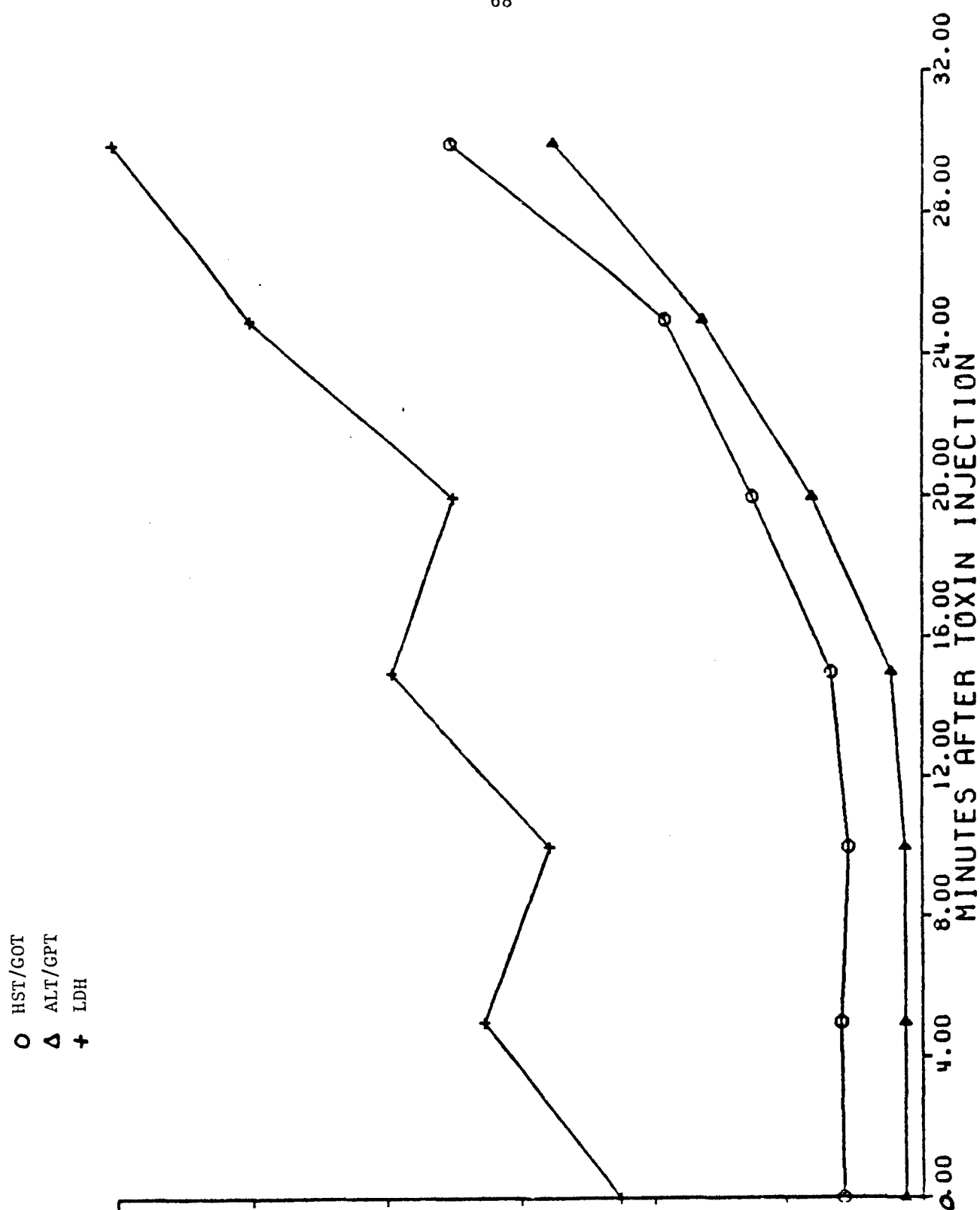


FIGURE 9 . BLOOD CHEMISTRY VALUES AFTER TOXIN-LR

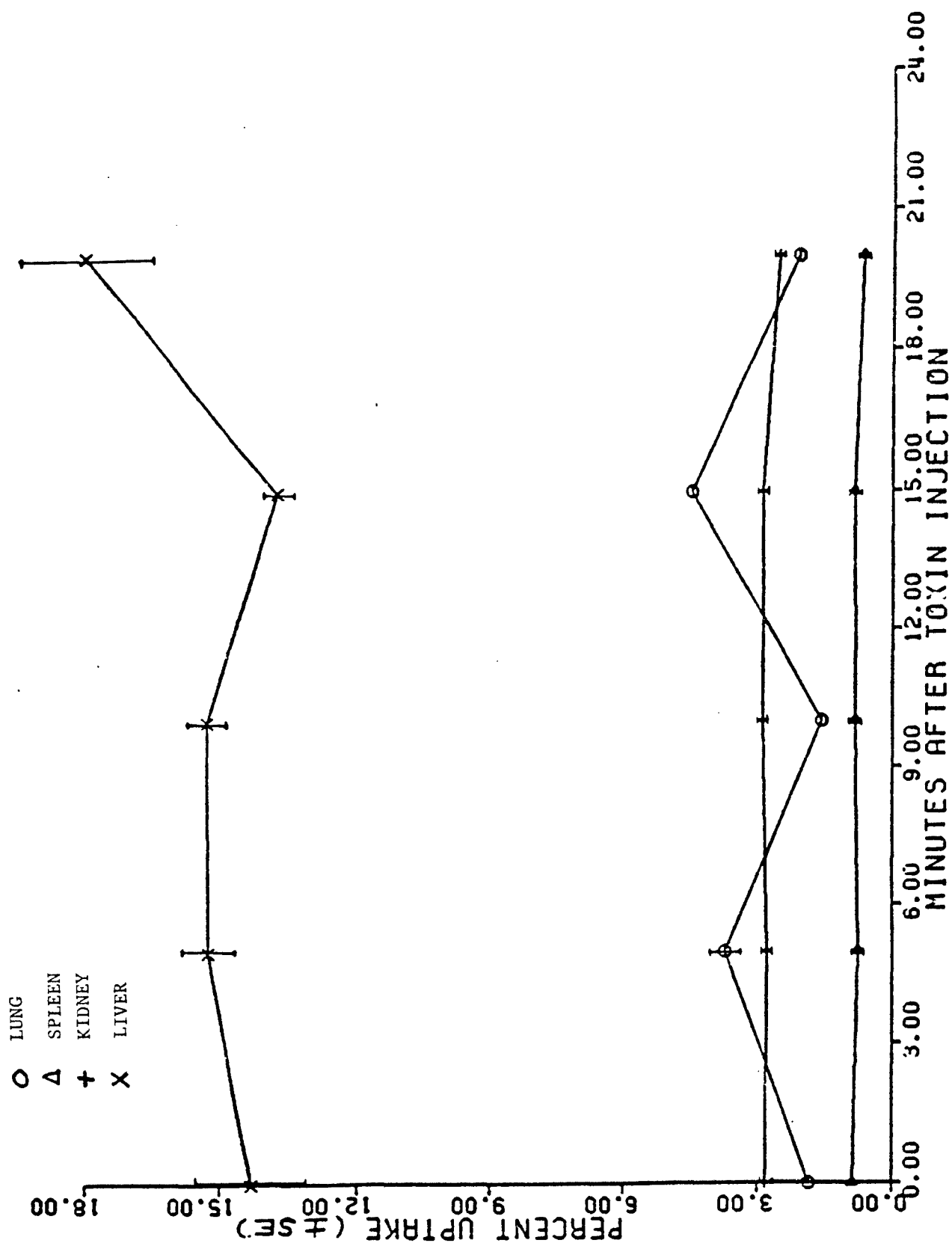


FIGURE 10. UPTAKE OF ALBUMIN-I125 AFTER TOXIN-LR



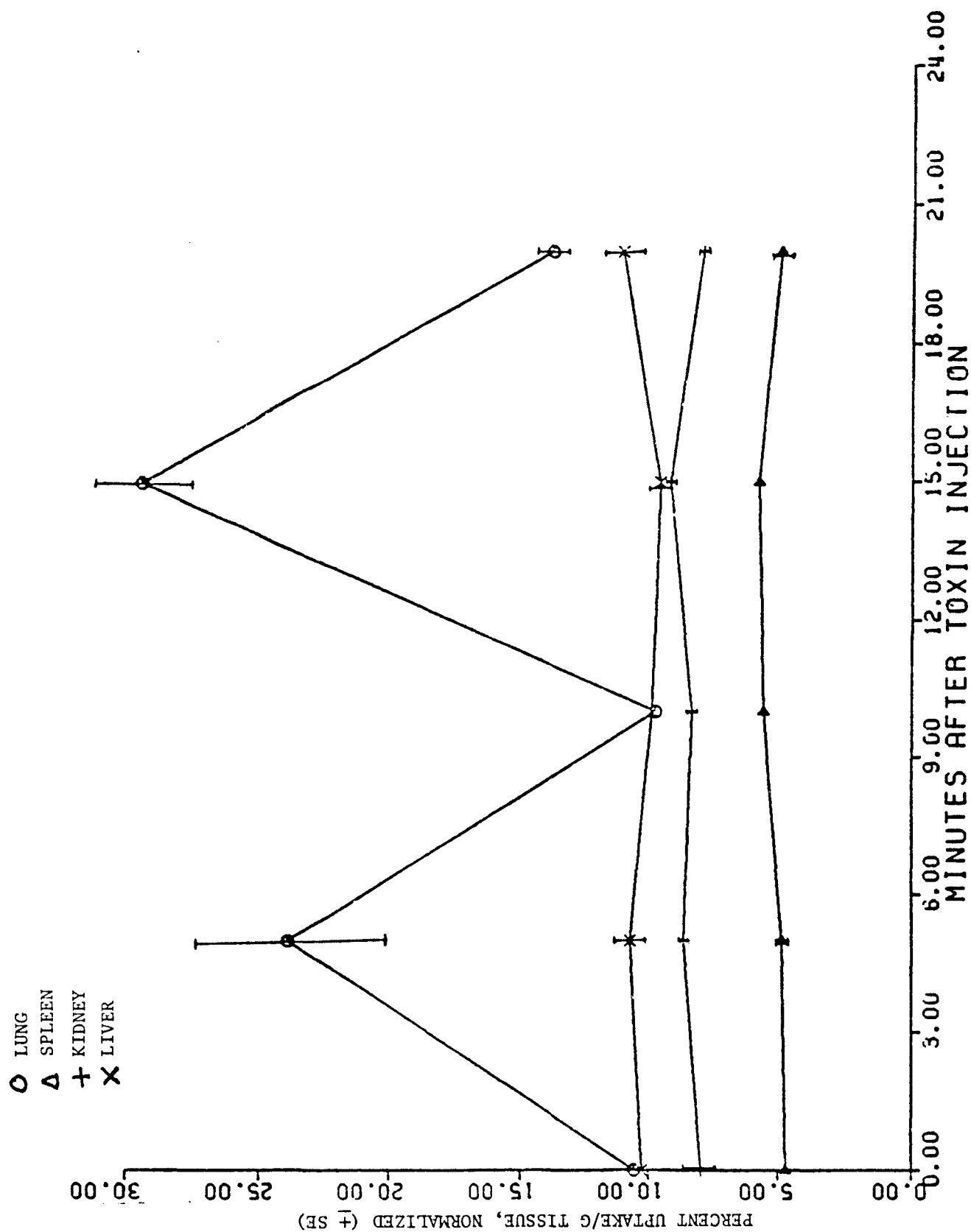


FIGURE II. UPTAKE OF ALBUMIN-1125 AFTER TOXIN-LR

FIGURE 12

20 MINUTES

15 MINUTES

10 MINUTES

5 MINUTES

CONTROL

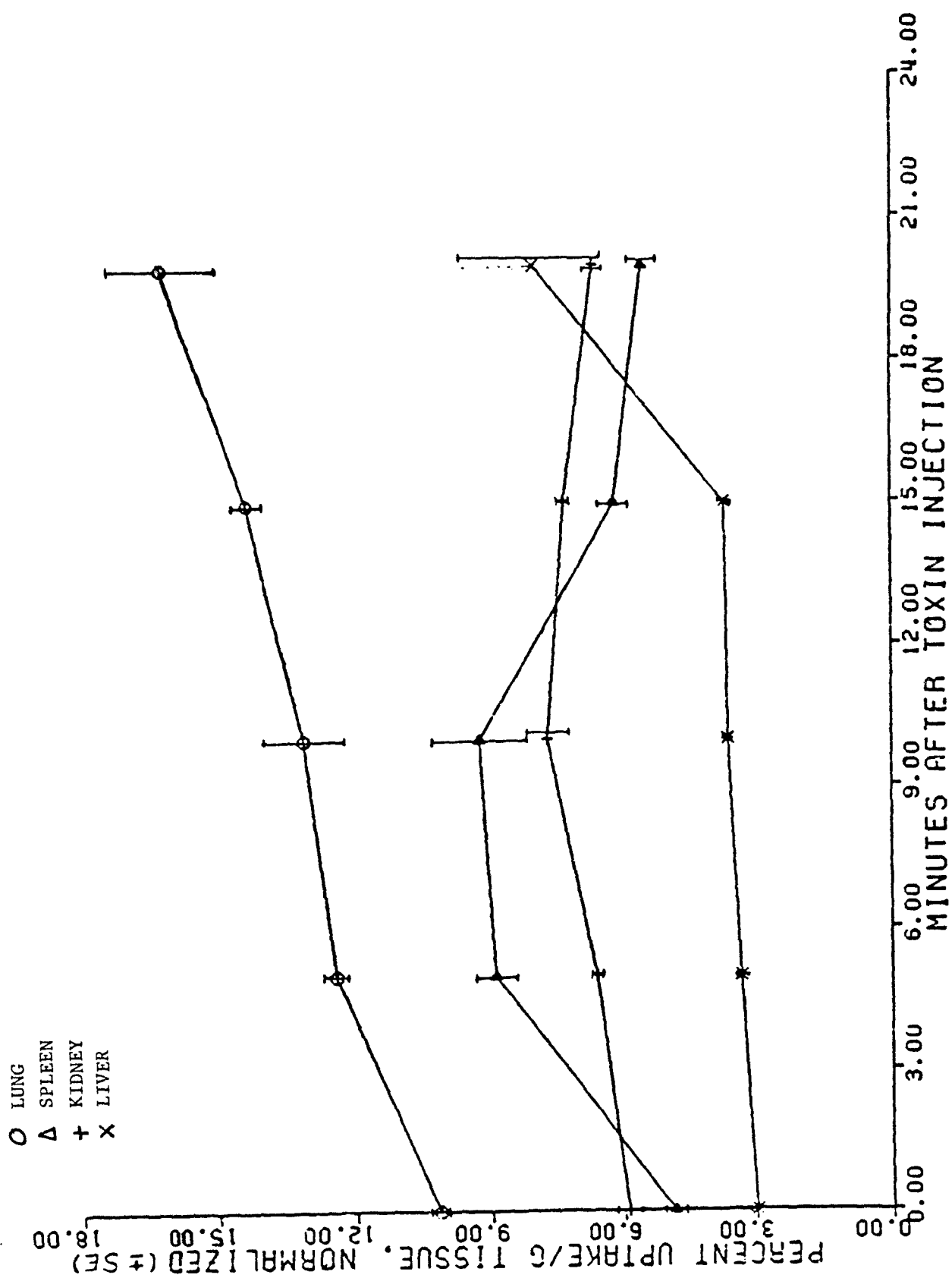


FIGURE 13. UPTAKE OF MRBC-CR51 AFTER TOXIN-LR



FIGURE 14

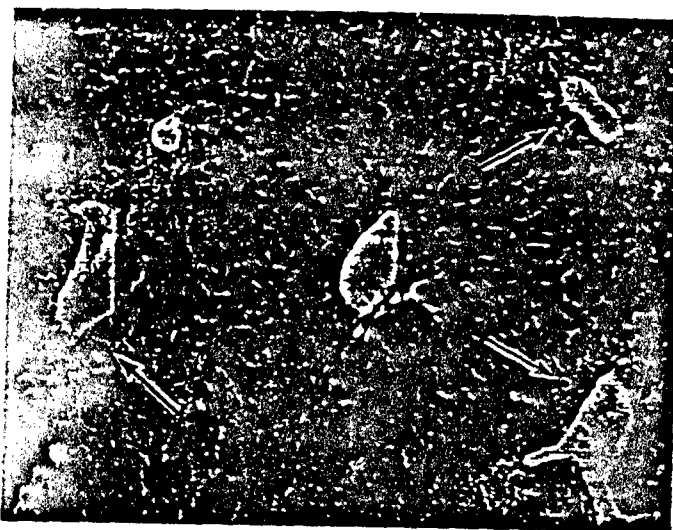


FIGURE 15

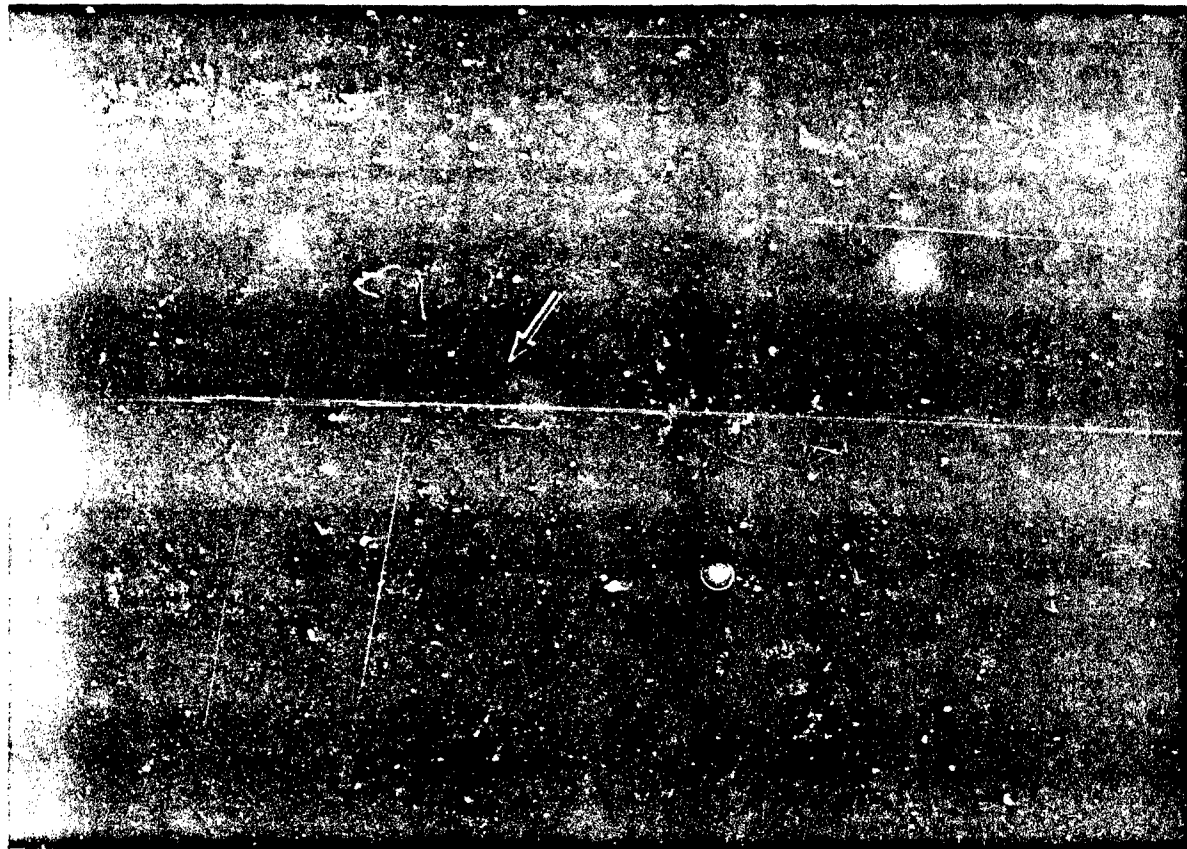


FIGURE 16

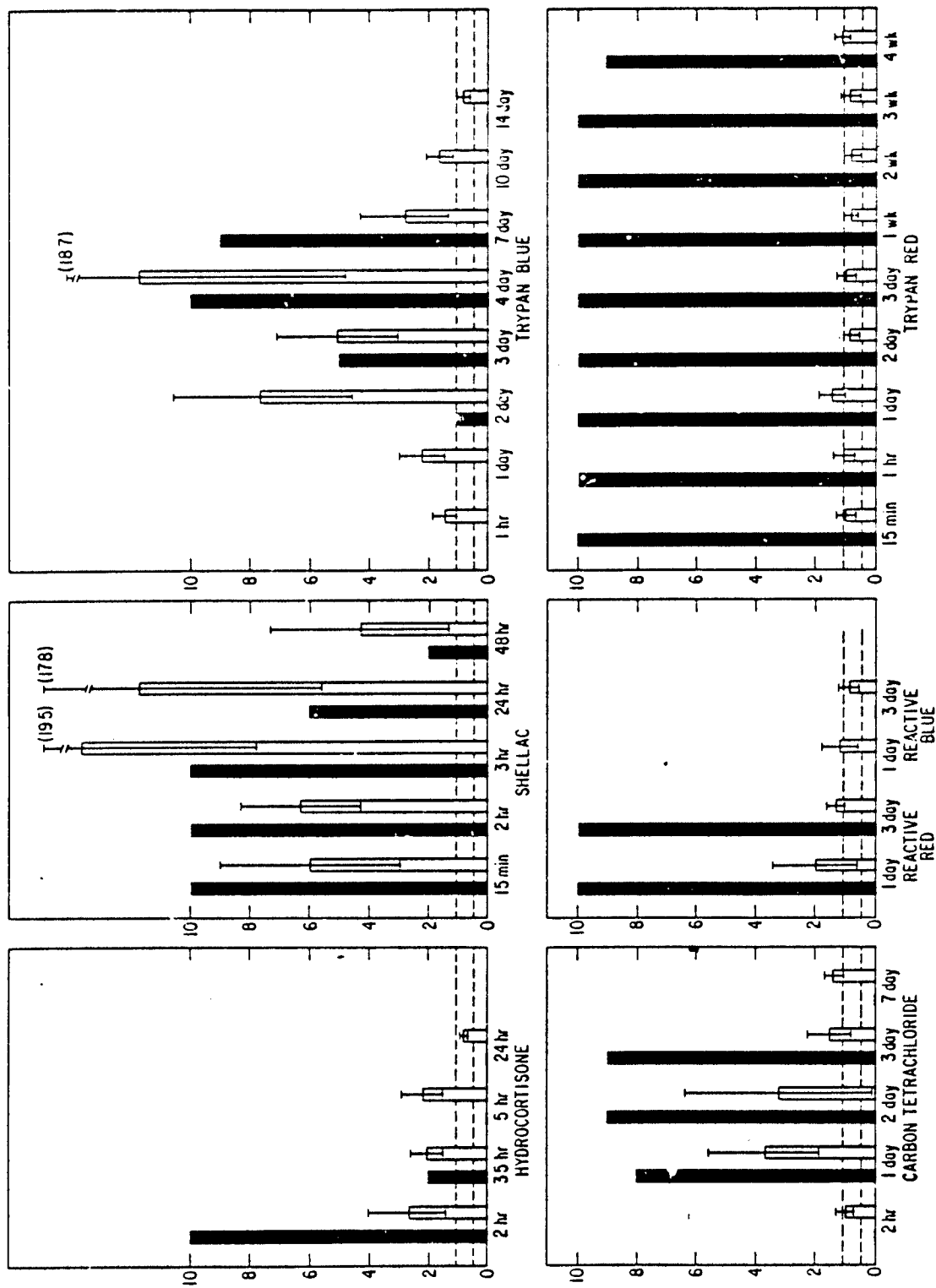


FIGURE 17